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Communication

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CD39 Is an Ecto-(Ca^{2+},Mg^{2+})-apyrase*

(Received for publication, February 15, 1996)

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CD39, a 70- to 100-kDa molecule expressed primarily on activated lymphoid cells, was previously identified as a surface marker of Epstein Barr virus (EBV)-transformed B cells. In this report, we show that an ecto-(Ca^{2+},Mg^{2+})-apyrase activity is present on EBV-transformed B cells, but not on B or T lymphomas. The coincidence between CD39 expression and ecto-apyrase activity on immune cells suggests that CD39 may be an ecto-apyrase. This supposition is supported by the observation that the amino acid sequence of CD39 is significantly homologous to those of several newly identified nucleotide triphosphatases. Finally, we show that CD39 indeed has ecto-apyrase activity by expression in COS-7 cells.

Ecto-(Ca^{2+},Mg^{2+})-apyrases (ecto-ATPases) or E-type ATPases (reviewed in Ref. 1) are glycoproteins that hydrolyze extracellular nucleotide tri- and/or diphosphates. These enzymes have a high specificity activity and are insensitive to inhibitors of P-type, F-type, and V-type ATPases (2–5).

Progress in the study of ecto-apyrases has been impeded by the lack of a specific inhibitor of enzymatic activity and by their low protein abundance. Moreover, most ecto-apyrases are inactivated by the detergents normally used to solubilize membrane-bound proteins. Recently, Handa and Guidotti (6) reported that a potato tuber (*Solanum tuberosum*) apyrase was similar in amino acid sequence to CD39, a human and mouse lymphoid cell antigen (7), and to several other newly identified NTPases,[1] including yeast guanosine diphosphatase (8), garden pea nucleotide triphosphatase (NTPase),[2] and *Toxoplasma gondii* NTPase (10, 11). CD39, a membrane glycoprotein, was originally identified as the major surface marker of EBV-transformed B lymphoblastoid cells (12). Later it was found primarily on activated immune cells (13–15). Although the function of CD39 is largely unknown, the similarity between CD39 and the NTPases suggested that CD39 might be an ecto-apyrase.

In this report, we show that EBV-transformed B lymphocytes have ecto-apyrase activity, whereas non-EBV-transformed B lymphocytes do not. Ecto-apyrase activities were also found associated with other immunocompetent cells, including cytotoxic T cells (16) and natural killer (NK) cells (4), but not with resting thymocytes and lymphomas (4, 17, 18). Both in our studies and those of other investigators, there is a coincidence between expression of CD39 and ecto-apyrase activity. All these clues lead to the hypothesis that CD39 may encode an ecto-apyrase. Finally, by transfection of COS-7 cells with CD39 cDNA, we show that CD39 indeed has ecto-apyrase activity.

MATERIALS AND METHODS

Reagents—Nucleotides, *N*-ethylmaleimide, ouabain, activated charcoal, DEAE-dextran, chloroquine, horseradish peroxidase-conjugated anti-mouse antibody, and nitrocellulose membrane were purchased from Sigma. [γ-^{32}P]ATP (triethylammonium salt) was purchased from DuPont NEN Research Products. RPMI 1640, penicillin/streptomycin, DMEM, and L-glutamine were purchased from Life Technologies, Inc. Anti-CD39 monoclonal antibody AC-2 (7, 12) was purchased from AMAC, Inc. (Westbrook, ME). Chemiluminescent reagents were purchased from Kirkegard & Perry Laboratories (Gaithersburg, MD).

Cell Cultures—EBV-transformed B-lymphoblasts from a patient with cystic fibrosis (GM7227A) were from the Coriel Institute for Medical Research (Camden, NJ) (19). Human T leukemia Jurkat cells, EBV-transformed B lymphoblast LG2 cells, murine T lymphoma BW5147 cells, and B lymphoma A20 and Daudi cells were kindly provided by Dr. Basya Ryalov (Dept. of Molecular and Cellular Biology, Harvard University). All immune cells were maintained in medium RPMI 1640. COS-7 cells were kept at 50–70% confluence in DMEM. All cultures were supplemented with 10% fetal bovine serum, 10 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Assay of Apyrase Activity—Apyrase activity was determined by measuring the amount of [^{32}P]P_i released from [γ-^{32}P]ATP after precipitation of nucleotides with activated charcoal (4, 20). Intact cells (1×10^5) or cell homogenates were added to buffer containing 20 mM HEPES-Tris (pH 7.4), 120 mM NaCl, 5 mM KCl, 1 mM EGTA, 0.3 mM ATP, and 0.8 μCi of [γ-^{32}P]ATP; the final volume was 200 μl. Cell extracts were obtained by homogenizing cells in the buffer with a Dounce homogenizer. The assay mixture was incubated at 37 °C for 20 min, and then the reaction was stopped by the addition of 0.5 ml of cold 20% (w/v) activated charcoal in 1.0 M HCl. The assay tubes were incubated on ice for 10 min and centrifuged at 10,000 × *g* for 10 min to pellet the charcoal. Aliquots (140 μl) of the supernatant containing the released [^{32}P]P_i were transferred to scintillation fluid, and radioactivity was measured by using a Beckman LS5801 liquid scintillation spectrophotometer. Alternatively, the apyrase activity was determined by measuring the inorganic phosphate released as described by Ames (21), except that the time for color development was 20 min at 37 °C. All assays were performed in triplicate and reported as the mean and standard deviation. The Ca^{2+}- or Mg^{2+}-stimulated apyrase was determined by subtracting values obtained with EGTA alone from those with 1.5 mM $CaCl_2$ or $MgCl_2$ plus chelator.

Reverse Transcription-PCR and Construction of Expression Plasmid—Total RNA was isolated from approximately 10^7 human EBV-transformed B lymphocytes (LG2) by the acid guanidinium thiocyanate method (22). One microgram of total RNA served as template for cDNA synthesis by avian myeloblastosis virus reverse transcriptase (Life Technologies, Inc.) for 60 min at 42 °C in the presence of 25 pmol of a specific 30-base oligonucleotide as primer in a total reaction of 20 μl (23). The reverse transcription primer was the same one as the antisense primer to be used in the subsequent PCR reaction. The whole reverse transcription mixture was then used as template in 35 cycles of amplification. The sense primer (5′-GCGAATTCTTATGGAAGATACAAAGGAGTC), with an *Eco*RI site at the 5′ end, contains a sequence identical with nucleotides 68–87 of human CD39 (7). The antisense primer (5′-GCTGAATTCGCTATACCATATCTTTCCAGA) is complementary to nucleotides 1581–1600 of the CD39 coding sequence, except that an *Eco*RI site is present. After amplification, the PCR product (1.6 kilobases) was subcloned into pCI-neo (Promega).

Expression of CD39 in COS-7 Cells and ATPase Assay—COS-7 cells

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[1] The abbreviations used are: NTPase, nucleotide triphosphatase; EBV, Epstein-Barr virus; NK, natural killer cells; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction.

[2] H. Hsieh and R. J. Roux, Direct submission, GenBank™ accession number Z32743.

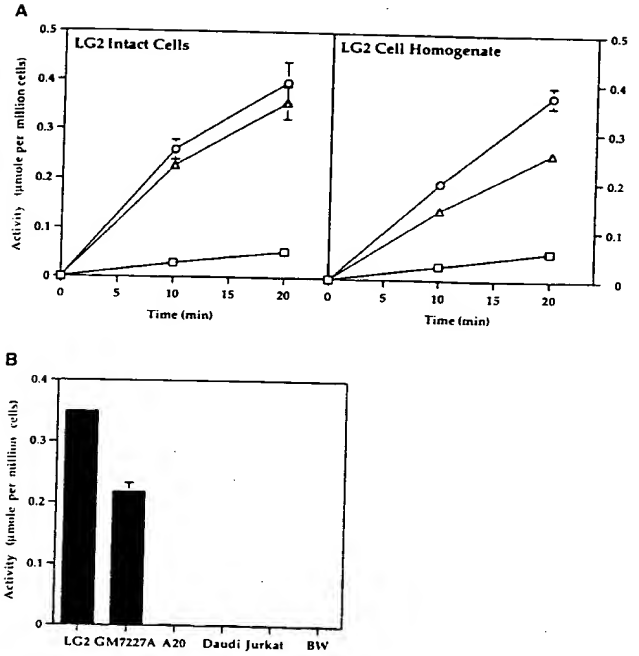

FIG. 1. **Ecto-(Ca^{2+}, Mg^{2+})-apyrase activity in EBV-transformed B cells.** *A*, time course of Ca^{2+}- and Mg^{2+}-stimulated apyrase activities of EBV- transformed B cell, LG2. Intact cells (*left panel*) or homogenate (*right panel*) were assayed at a concentration of 5×10^5 cells/ml in the presence of 1 mM NaN_3, 0.5 mM Na_3VO_4, and 1 mM EGTA without added Ca^{2+} or Mg^{2+} (□), or with 1.5 mM Ca^{2+} (△), or with 1.5 mM Mg^{2+} (○). *B*, ecto-(Ca^{2+})-apyrase activities of EBV-transformed B cells (LG2 and GM7227A), non-EBV-transformed B cells (A20 and Daudi), and T lymphomas (Jurkat and BW5147), assayed as described in *A*.

were transfected with cDNA for CD39 or with the pCI-neo vector alone by the DEAE-dextran method (24). The cells were seeded for 2 days before transfection at a density of 1700 cells per cm^2. On the day of transfection, the cells were washed twice with DMEM. Then, DMEM containing 10% Nuserum, 0.4 mg/ml DEAE-dextran, and chloroquine (100 μM) was added. DNA was added at a concentration of 1.25 μg/ml, and the cells were incubated for 4 h at 37 °C with 5% CO_2. Afterward, the cells were washed once with DMEM, shocked for 2 min in 10% dimethyl sulfoxide in phosphate-buffered saline and washed twice with phosphate-buffered saline. The cells were incubated in DMEM containing 10% fetal bovine serum at 37 °C with 5% CO_2. After 2 to 3 days, cells were detached by adding 10 mM EDTA and then incubated at 37 °C for 20 min. The detached cells were pooled, centrifuged, washed twice with assay buffer, and assayed for calcium-dependent ecto-apyrase activity.

Immunoblots—Cells extracts (10 or 50 μg) were resolved by 9% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes for 2 h at 500 mA. Membranes were washed in rinse buffer (phosphate-buffered saline with 3% Tween 20) at room temperature for 15 min and incubated overnight at 4 °C with AC2 anti-CD39 mAb (0.2 μg/ml) in rinse buffer. The membranes were washed three times with rinse buffer at room temperature and were incubated with a 1:2000 dilution of horseradish peroxidase-conjugated anti-mouse antibody in rinse buffer for 1.5 h at room temperature. After three washes, membranes were developed with chemiluminescent reagents, and the emitted light was recorded by x-ray film.

RESULTS

Ca^{2+} and Mg^{2+}-stimulated Apyrase Activity of EBV-transformed B Lymphocytes—Human EVB-transformed B lymphocytes LG2 and GM7227A were incubated in the standard reaction buffer with [γ-^{32}P]ATP in the presence of 1 mM NaN_3 and 0.5 mM Na_3VO_4 for 20 min. After charcoal precipitation of the nucleotides, the released [γ-^{32}P]P_i in the supernatant represents the apyrase activity. The hydrolysis of [γ-^{32}P]ATP was linear for at least 25 min, and no radioactivity was released above background levels in assays containing no cells. Fig. 1*A* (*left panel*) shows the time course of Ca^{2+}- and Mg^{2+}-stimulated apyrase activity of intact LG2 cells. In the absence of a divalent cation, almost no hydrolysis of ATP was detected, whereas both Ca^{2+} and Mg^{2+} stimulated the activity. At the end of the incubation, cells were intact (>90%) as demonstrated by exclusion of trypan blue. Disruption of LG2 cells by homogenization in a Dounce homogenizer did not increase either Ca^{2+}- or Mg^{2+}-apyrase activity (*right panel*), suggesting that the (Ca^{2+},Mg^{2+})-apyrase activity was probably associated with the external surface of the plasma membrane. The release of P_i from EBV-transformed LG2 and GM7227A cells was 349 ± 8 and 218 ± 10 nmol per million cells per 20 min, respectively. To examine whether this hydrolytic activity was due to secreted enzymes or an intracellular enzyme released through "leaky" membrane, cells were incubated for 30 min in assay buffer without ATP and pelleted. Apyrase activity in the supernatant was less than 5% of the total apyrase activity, whereas the cell pellet contained more than 90% of the total apyrase activity. These results indicated the presence of a membrane-associated ecto-apyrase in EBV-transformed B lymphocytes.

TABLE I
Characterization of ecto-apyrase activity

Control ecto-apyrase activity was determined using 1×10^5 LG2 cells in the standard solution as described under "Materials and Methods." Ca^{2+}-stimulated apyrase activities in the presence of inhibitors were determined at the end of a 20-min incubation by subtracting values obtained with EGTA alone from those with calcium plus chelator.

Inhibitors	% control
Control	100 ± 3
Ouabain (1 mM)	86 ± 4
VO_4^- (0.5 mM)	77 ± 3
N_3^- (1 mM)	75 ± 3
N-Ethylmaleimide (10 mM)	95 ± 3
F^- (10 mM)	85 ± 2

To further characterize the observed apyrase activity, assays were done with human T leukemia Jurkat cells (ecto-apyrase-deficient (18)), African Burkitt lymphoma Daudi cells, murine T lymphoma BW5147 cells, and murine B lymphoma A20 cells. The apyrase activities of these cells were not statistically elevated above the background level (Fig. 1*B*).

Characterization of Ecto-apyrase Activity—Table I shows that inhibitors of P-type plasma membrane ATPases (ouabain and vanadate), V-type vacuolar ATPase (N-ethylmaleimide), and F-type mitochondrial ATPase (azide), did not significantly inhibit the EBV-transformed LG2 ecto-apyrase activity. Furthermore, 10 mM fluoride (a phosphatase inhibitor) was not inhibitory. The nucleotide specificity of the LG2 (Ca^{2+},Mg^{2+})-apyrase activity is shown in Table II. The relative hydrolysis rates of the nucleotide triphosphates is about the same, while the rate with ADP is about 60% of that with ATP. No phosphate was released from AMP (substrate of 5′-nucleotidase) and *p*-nitrophenyl phosphate (substrate of alkaline phosphatase). These results suggest that the (Ca^{2+},Mg^{2+})-apyrase activity of EBV-transformed LG2 cells is typical of an E-type ATPase.

Coincidence of CD39 Expression Pattern and Ecto-apyrase Activity in Immunocompetent Cells—CD39 was originally identified as a surface antigen of EBV-transformed lymphoblastoid cells (12). CD39 is not encoded by the EBV genome, but is a host gene. Epitope-tagging and topologic analysis indicated that CD39 has a large extracellular loop between short intracellular N and C termini. The large extracellular loop was similar in amino acid sequence to yeast guanosine phosphatase, indicating that CD39 may encode an ecto-enzyme (7). Later, CD39 was found primarily on activated immune cells, but was absent from resting thymocytes, lymphocytes, and lymphomas. In both our studies and those of other investiga-

TABLE II
Substrate specificity of ecto-(Ca^{2+})-apyrase

The apyrase activity was determined by measuring the inorganic phosphate released as described by Ames (21), except that the time for color development was 20 min at 37 °C. The LG2 cells were incubated in the standard solution with 1 mM NaN_3 and 0.5 mM Na_3VO_4. The reaction was started by adding the nucleotides to a final concentration of 1 mM and incubating at 37 °C. Ca^{2+}-stimulated apyrase activity was determined at the end of a 20-min incubation by subtracting values obtained with EGTA alone from those with calcium plus chelator.

Substrates	% control
ATP	100 ± 4
GTP	89 ± 3
CTP	75 ± 3
dATP	95 ± 2
ADP	58 ± 2
AMP	0 ± 1
p-Nitrophenyl phosphate	1 ± 1

tors, there is a coincidence between the expression pattern of CD39 and ecto-apyrase activity (Table III). CD39 was only found in cells with ecto-apyrase activity, suggesting that CD39 may encode an ecto-apyrase.

Demonstration of CD39 as an Ecto-apyrase—Since detergent solubilization is a major problem in purification of many ecto-apyrases and LG2 cells lost more than 90% of the ecto-apyrase activity after solubilization with $C_{12}E_9$ or Triton X-100, we decided to test whether CD39 encoded an ecto-apyrase by expression. Human CD39 cDNA was amplified by reverse transcription-PCR and subcloned into the mammalian expression vector pCI-neo. COS-7 cells transfected with CD39 recombinant cDNA had about 5.4-fold higher Ca^{2+}-ATPase activity (309 ± 27.6 nmol/mg/h) than cells transfected with vector alone (57.6 ± 4.8 nmol/mg/h) (Fig. 2*A*). This activity was not inhibited by P- or V-type ATPase inhibitors, since 1 mM NaN_3 and 0.5 mM Na_3VO_4 were included in the reaction buffer. Cells extracts (50 μg) of both preparations were examined for the presence of CD39 by Western analysis (Fig. 2*B*). Cells transfected with recombinant CD39 cDNA had 5-fold more CD39 protein (*lane 2*) as compared with cells transfected with vector alone (*lane 1*), estimated by comparison with the amount of CD39 protein in 10 μg (*lane 3*) and 50 μg (*lane 4*) of protein from extracts of LG2 cells.

TABLE III
Distribution of ecto-apyrase activity and CD39 among immunocompetent cells

Cell lines	CD39	Ecto-apyrase
EVB-transformed B cells		
LG2	+	+
GM7727A	+	+
B cell lines		
A20	−	−
Daudi	−[a]	−
Raji	−[a,b]	−[c,d]
NK cells		
Unactivated	−[a]	ND[e]
Activated	+[a]	+[c]
T cell clones (activated)		
Cytotoxic cells	+[a]	+[c]
Helper cells	+[a]	ND[e]
T cell lines (unactivated)		
BW5147	−	−
Jurkat	−	−
MOLT3	−[f]	−[g]
CEM	−[b,f]	−[g]

[a] Ref. 13.
[b] Ref. 12.
[c] Ref. 4.
[d] Ref. 18.
[e] Not determined.
[f] Ref. 14.
[g] Ref. 17.

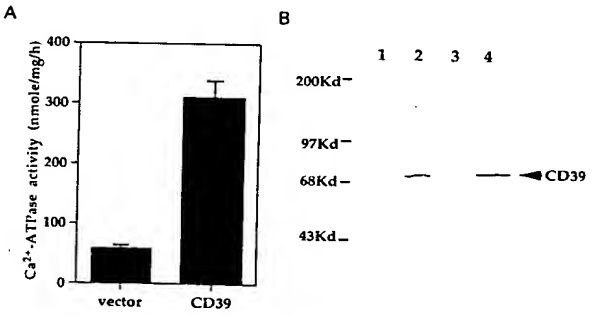

FIG. 2. **CD39 encodes an ecto-apyrase.** *A*, Ca^{2+}-stimulated apyrase activity of COS-7 cells with expression vector pCI-neo or CD39 recombinant DNA. *B*, immunoblot analysis of CD39 in total cell extracts of COS-7 cells transfected with vector (*lane 1*, 50 μg) or CD39 recombinant DNA (*lane 2*, 50 μg) and of LG2 Cells (*lane 3*, 10 μg; *lane 4*, 50 μg) as described under "Materials and Methods."

DISCUSSION

This paper reports that CD39 is an ecto-apyrase. Four lines of evidence support this conclusion. First, the amino acid sequence of CD39 is significantly homologous to several newly identified NTPases. Second, the coincidence of the expression pattern of CD39 and ecto-apyrase activities on immunocompetent cells is consistent with the hypothesis that CD39 is an ecto-apyrase. Third, ecto-apyrase activities were found on EBV-transformed B lymphocytes that express CD39 as a major surface marker (12). No ecto-apyrase activity was found on non-EBV-transformed B lymphoma cell lines. Finally, expression of CD39 cDNA in COS-7 cells increases their ecto-apyrase activity at least 5-fold.

CD39 is expressed on activated NK cells, B cells, and T cell clones, but is not expressed by resting blood T, B, or NK cells, neutrophils, or monocytes. CD39 expression in lymphoid tissue is primarily limited to mantle zone and paracortical lymphocytes, macrophages, and dendritic cells and is generally absent from germinal centers (13). Because CD39 encodes an ecto-apyrase, the restriction of its expression to activated lymphoid cells and in anatomical sites of ongoing B cell differentiation suggests that ecto-apyrase activity may play an important role in immune responses.

One possible role of an ecto-apyrase in immune cells is to protect them from potential lytic effects of extracellular ATP released by their target cells (16, 25). Extracellular ATP can induce cell death in many immune cells and a few tumor cell lines. ATP^{4-} binds to P_2z purinergic receptors and causes the opening of a nonselective membrane pore, which has a molecular cut-off of approximately 0.9 kDa (26). By measuring ATP-induced uptake of extracellular markers of less than 0.9 kDa, P_2z receptors were found on most immune cells and some tumor cell lines. Because of the relative high dose of extracellular ATP needed for P_2z receptor activation (half-maximal effective concentration, EC_{50}, varies from 100 μM to 1 mM), Di Virgilio (25) proposed that "leakage ATP" from the cytoplasm of stressed or injured cells was the most likely source for extracellular ATP and such an event was likely to occur at the site of inflammatory or immune responses. Hydrolysis of extracellular ATP by plasma membrane ecto-(Ca^{2+},Mg^{2+})-apyrases leads to closure of the P_2z receptor pores (27). Interestingly, persistent treatment with low doses of interleukin-2 can induce CD39 expression in T cell and NK cell *in vivo* (15); in mouse splenocytes this treatment causes resistance to extracellular ATP (28–30).

Ecto-apyrase activities have been found not only in the immune system, but also in many other tissues. The broad distribution of ecto-apyrase activity suggests that it may have

essential functions. Unfortunately, the molecular structure and function of ecto-apyrases in those tissues are largely unknown. At least three hypotheses for the roles of ecto-apyrase have been suggested. One function might be hydrolysis of ATP and other nucleotides to terminates their roles as P_2-purinergic ligands (reviewed in Ref. 31). Secondly, ecto-apyrases may have a principal role in the formation of AMP by hydrolysis of both ADP and ATP (32). Extracellular AMP can be converted by ecto-5′-nucleotidase to adenosine, a ligand of P_1 receptors (33, 34). Both ATP and adenosine modulate a variety of key cellular processes in many tissues and organs. A third possibility is the cooperation of ecto-apyrase and 5′-nucleotidase activities with a sodium-dependent adenosine cotransporter in rat liver canalicular membranes to convert extracellular ATP to intracellular adenosine leading to conservation of the purine (9). We wonder whether CD39 or similar genes may correspond to ecto-apyrase activities in nonimmune tissues. Although CD39 was also found in endothelium (13) and human placenta,[3] the expression pattern of CD39 in other nonimmune tissues has not been established.

In summary, our results show the CD39 encodes an ecto-(Ca^{2+},Mg^{2+})-apyrase. This is the first identification of a mammalian ecto-apyrase gene. Further analysis of CD39 may help in the understanding of the structure and function of other ecto-apyrases.

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